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Inhibition of Settlement and Metamorphosis of the Ascidian *Herdmania curvata* by Non-geniculate Coralline Algae

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Abstract. The surfaces of non-geniculate coralline algae (NCA) are known to induce the settlement and metamorphosis of disparate marine taxa. In this study we investigate the responsiveness of larvae of *Herdmania curvata* (Ascidiacea: Stolidobranchia) to three species of NCA (*Neogoniolithon brassica-florida, Hydrolithon onkodes,* and *Lithothamnium prolifer*) that cohabit the slope and crest of Heron Reef, Great Barrier Reef. *H. curvata* larvae were first exposed to these NCA at or within 2 h of hatching, which is 1 to 2 h prior to attaining competence, and then cultured continuously with the NCA for 12 to 14 h. Rates of settlement and metamorphosis of *H. curvata* cultured in laboratory chambers in the presence of the different NCA were significantly lower than spontaneous rates in seawater. The limited settlement in treatments containing NCA were confined entirely to the chamber periphery, and settlement never occurred on the surface of the NCA. The inhibitory effect was dose-dependent and was stronger in *N. brassica-florida* and *H. onkodes* than in *L. prolifer*. Larvae that did not settle in treatments with NCA had rounded anterior trunks and, in extreme cases, kinked tails with rounded and dissociated tail muscle cells. In some individuals, we observed the anterior chemosensory papillae being sloughed off the larval body. Morphological analysis of trunk ectodermal and mesenchymal nuclei of larvae cultured in the presence of the NCA revealed that general necrotic cell death was occurring. Importantly, *H. curvata* larvae that were exposed to NCA could not subsequently be induced to metamorphose in KCl-elevated seawater, whereas larvae not exposed to NCA metamorphosed at high rates in KCl-elevated seawater.

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Introduction

Marine invertebrate larvae traverse a complex chemical seascape and can encounter factors that promote or deter settlement and metamorphosis. The unique and sometimes opposite responses of different larvae to chemicals emanating from or on the surface of a particular substratum has been attributed to differences in larval chemosensory capabilities (reviewed in Hadfield, 1986; Hadfield and Pennington, 1990; Morse, 1990, 1993; Pawlik, 1992; Leitz, 1997) and developmental states (Trapido-Rosenthal and Morse, 1986; Degnan and Morse, 1995). At different ages, competent larvae of a given species can respond differentially to a particular inductive cue, either by settling or metamorphosing at different rates (*e.g.,* Coon *et al.,* 1990; Degnan *et al.,* 1995).

Despite the apparent diversity of larval chemosensory systems, disparate marine invertebrate taxa often respond in a similar manner to particular substrata. Bacterial films on both biotic and abiotic substrata induce settlement in a wide range of marine invertebrates (reviewed in Pawlik, 1992; Johnson *et al.,* 1997), although several species are inhibited from settling by bacteria or their products (*e.g.,* Maki *et al.,* 1988, 1992) or show no response to bacteria (*e.g.,* Keough and Ramondi, 1995). In contrast, in most cases the surfaces of sessile marine invertebrates such as sponges, bryozoans, and ascidians either contain inhibitors or do not provide morphogenic cues to induce the settlement of larvae (reviewed in Davis *et al.,* 1989; Pawlik, 1992). A finer scale of analysis reveals greater complexity in larval/substratum interactions. For example, in the case of microbial films,

larvae of some species respond to a variety of bacterial genera (*e.g.,* Fitt *et al.,* 1989), whereas others apparently require particular strains or communities (*e.g.,* Wilson, 1955; Kirchman *et al.,* 1982; see also Johnson and Sutton, 1994). Similarly, a particular strain may induce settlement in some species but not others (Tritar *et al.,* 1992) or even inhibit settlement in some species but promote settlement in others (*cf.* Kirchman *et al.,* 1982; Maki *et al.,* 1990, 1992).

The range of interactions between invertebrate larvae and the surface of non-geniculate coralline algae (NCA) is similar to that exhibited between larvae and microbial films. NCA induce settlement, metamorphosis, or both in a variety of echinoderms (*e.g.,* Rowley, 1989; Johnson *et al.,* 1991), molluses (*e.g.,* Barnes and Gonor, 1973; Heslinga, 1981; Rumrill and Cameron, 1983; Morse and Morse, 1984; Moss and Tong, 1992), annelids (*e.g.,* Gee and Knight-Jones, 1962; Gee, 1965), and coelenterates (*e.g.,* Harrigan, 1972; Sebens, 1983a, b; Morse *et al.,* 1988), but do not provide a morphogenic signal for several species of tubicolous annelids (*e.g.,* De Silva, 1962; Gee and Knight-Jones, 1962; Jensen and Morse, 1984). Among species whose larvae are induced by NCA, the specificity of the interaction covers a spectrum from species that manifest specificity for a particular species of NCA (Gee and Knight-Jones, 1962; Gee, 1965; Johnson *et al.,* 1991), to those requiring contact with any of a variety of NCA (Morse *et al.,* 1988), to those requiring a cue from NCA or any of a variety of other substrata (Harrigan, 1972; Heslinga, 1981; Hahn, 1989).

In this study we investigate the responsiveness of larvae of the tropical solitary ascidian *Herdmania curvata* (previously known as *H. momus* "Heron Reef" or *H. momus* forma *curvata;* see Degnan and Lavin, 1995) to three species of NCA, *viz. Neogoniolithon brassica-florida* (previously known as *N. foslei*), *Hydrolithon onkodes* (previously known as *Pololithon onkodes*), and *Lithothamnium prolifer. H. curvata* and these NCA inhabit the slope and crest of reefs along the Great Barrier Reef. Since larval ontogeny and the cellular basis of morphological change are understood in solitary ascidians to a greater extent than in most marine invertebrates (reviewed in Satoh, 1994), and the developmental and morphogenetic processes that regulate the attainment of competence, settlement, and metamorphosis have been documented in *H. curvata* and other ascidians (Cloney, 1982; Torrence and Cloney, 1983; Degnan *et al.,* 1996, 1997; Eri *et al.,* 1999), this tropical ascidian is a useful model for investigating the inductive and inhibitory activities of various substrata. Settlement and metamorphosis can be induced rapidly in *H. curvata* by a range of artificial and natural cues, with most competent larvae initiating metamorphosis within 1 h of contact with the inductive signal (Degnan *et al.,* 1997). In addition, *H. curvata* larvae spontaneously settle and metamorphose in axenic cultures at a slower rate, such that up to 80% will be settled within 24 h of hatching. This "spontaneous" settlement and metamorphosis facilitates the analysis of inhibition of these processes.

Here we demonstrate that all three species of NCA inhibit settlement and metamorphosis of *H. curvata* in the laboratory. In the presence of the algae, settlement rates were much less than rates of spontaneous settlement in the absence of the NCA, and the limited settlement in the presence of NCA was confined to the periphery of the culture chamber and never occurred on the surface of the plant. Morphological analysis of larvae cultured in the presence of the NCA revealed that the NCA were toxic to the larvae, resulting in necrotic cell death and sloughing of the anterior chemosensory papillae. This toxic inhibition prevents these larvae from subsequently responding to a strong artificial inductive cue (40 m*M* KCl-elevated seawater; Degnan *et al.,* 1997). This result contrasts with that observed for some echinoderm larvae, which are readily induced to settle and metamorphose on contact with some of these NCA species (Johnson *et al.,* 1991; Johnson and Sutton, 1994).

Materials and Methods

Collection, maintenance, and cultivation of Herdmania curvata *and coralline algae*

Gravid *Herdmania curvata* and pieces of the non-geniculate coralline algae (NCA) *Neogoniolithon brassica-florida, Hydrolithon onkodes,* and *Lithothamnium prolifer* were collected from the reef crest and slope of Heron Reef, Great Barrier Reef, Australia (23° 27′ S; 151° 55′ E), and maintained separately in flowing, ambient seawater on site. NCA were collected by chiselling the algae from the surface of coral rubble.

H. curvata eggs were fertilized by pooling the gametes from at least three individuals into 0.2-μm-filtered seawater (FSW), and embryos were cultured in FSW as described in Degnan *et al.* (1996). Thin (1–2 mm) shards of NCA were prepared by chipping away most of the coral rubble from underneath the algae. Only shards free of macroscopic fouling organisms were used in the experiments.

Settlement experiments

Three experimental designs were used to examine the settlement response of *H. curvata* to the NCA: (1) larvae were presented with all species of NCA simultaneously (the "choice" experiment); (2) larvae had access to only a single species of NCA (the "no choice" experiment); and (3) a "no choice" design in which a dose-dependent response was examined.

Choice experiment. A variable number of small shards of each species of NCA (total epithallial area of *ca.* 1.5–3 cm^2 for each species) were placed in the center of 100-mm-diameter polycarbonate chambers containing 200 ml of seawater. Shards of each species were arranged so that the

epithallial surfaces faced both upwards and downwards. *H. curvata* embryos were mass cultured in FSW at 24°C until hatching, which is about 10 h after fertilization (Degnan *et al.*, 1996); newly hatched larvae require another 3–4 h of development to attain competence (Degnan *et al.*, 1997). Pre-competent *H. curvata* larvae (10–12 h old) were transferred from the mass culture to chambers (mean number of larvae *ca.* 130 per chamber) containing NCA and incubated at the ambient water temperature (22°–24°C) for 12–14 h. For controls, larvae were added to chambers containing seawater only. The experiment was repeated on consecutive days with different batches of larvae. Since the results of the two experiments were similar, data were combined for presentation, yielding a total of 15 replicate chambers of each of the NCA and control treatments.

After incubation, the NCA were removed from the chamber, placed in a shallow dish containing seawater, and inspected microscopically for settled *H. curvata* on the epithallial and rubble surfaces. The seawater in the chamber was filtered through a 60-μm mesh, and the chamber was gently washed with an additional 100 ml of seawater, which was also filtered. The chamber and the mesh were inspected and scored for larvae, settled postlarvae, and unsettled postlarvae (*i.e.*, larvae that underwent metamorphosis but not settlement).

After being scored, specimens of *H. curvata* were either inspected microscopically, fixed for histological analysis, or artificially induced to metamorphose (see below). Light microscopy was performed on living individuals with an Olympus BH-2 light microscope fitted with Nomarski optics; photomicrographs were obtained on an Olympus C-35AD-2 camera attached to this microscope.

No choice experiment. This experiment was conducted similarly to the choice experiment outlined above except that each chamber contained only a single species of NCA. The mean number of pre-competent larvae added to each chamber was 110, and there were 10 replicate chambers of each treatment containing NCA, and 9 replicate controls containing seawater but no NCA.

Dose-dependency (no choice): To determine the nature of any dose-dependency in the response to NCA, larvae were added to polycarbonate tissue culture vessels (35-mm diameter ×10-mm depth) containing 5 ml of seawater and either a single small, medium, or large (surface area of base of chamber respectively 5%–15%, 25%–35%, and 50%–75%) shard of NCA. Each chamber contained a single species of NCA, and about 30 newly hatched larvae (*i.e.*, 3 h before development of competence) were added to each. There were 6 replicate chambers per treatment, to give a total of 3 species NCA × 3 size classes × 6 replicates = 54 chambers containing NCA, and 6 control chambers containing larvae but no NCA, to yield a total of 60 chambers in the experiment.

After 12–14 h of incubation, the following categories of larvae were scored: settled on NCA (epithallus), settled on the rubble surface, settled on the sides of the chamber, metamorphosed but not settled, and neither metamorphosed nor settled. Larvae that had not yet metamorphosed or settled in chambers containing large shards of *L. prolifer* and the smallest shards of *N. brassica-florida* and *H. onkode* were transferred to 40 m*M* KCl-elevated FSW (see below).

Artificial induction of metamorphosis

To determine whether *H. curvata* larvae were competent to metamorphose after exposure to the NCA, we transferred 20 larvae that had been in the presence of large shards of *L. prolifer*, small shards of *H. onkodes* or *N. brassica-florida*, or seawater to new sterile 35-mm polycarbonate chambers containing 5 ml of 40 m*M* KCl-elevated FSW; this was performed in triplicate for each treatment (*i.e.*, 20 larvae × 3 replicates), and all larvae were derived from the same fertilization. KCl-elevated FSW is normally a potent artificial inducer of metamorphosis (Degnan *et al.*, 1997). Because most larvae that were previously exposed to seawater had metamorphosed by the time of the transfer, additional cultures were established as described in the above section to ensure that 60 larvae were transferred. Untreated control larvae were also transferred to FSW to control for the transfer process (an additional 60 larvae).

At 1-h intervals after transferral of larvae, each chamber was scanned to record the number of larvae that were undergoing metamorphosis. *H. curvata* larvae were considered to have initiated metamorphosis when the tail was at least 50% resorbed (Degnan *et al.*, 1996, 1997).

Histology and microscopy

Larvae that had been cultured in seawater or in the presence of the NCA were fixed in 4% (w/v) paraformaldehyde in 100 m*M* Hepes (pH 6.9), 2 m*M* $MgSO_4$, 1 m*M* EGTA for at least 3 h and stained with 2 mg/ml propidium iodide for 1 h as described in Hinman and Degnan (1998). Larvae were examined on a BioRad 600 laser confocal scanning microscope, exciting samples with 488-nm laser light and monitoring emission at 515 nm.

Statistics

Since there was no settlement on the epithallial surface of any NCA in the choice and no choice experiments, settlement rates on the chamber sides only were compared among NCA and control treatments with one-way Model I ANOVA. Similarly, in the dose-dependent experiment, there was little (if any) settlement on NCA surfaces; therefore, total settlement (on NCA and the plastic sides of chambers) was compared among the different "doses" (= size classes, 3 levels) and species (3 levels) using Model I two-way ANOVA. For this experiment the proportion of larvae neither settled nor metamorphosed and the proportion

metamorphosed but not settled were analyzed in separate Model I two-way ANOVAs. All *a posteriori* multiple range tests used Tukey's HSD criteria ($P = 0.05$).

For all analyses, the relationship between group means and standard deviations was examined to determine the appropriate transformation (if any) to stabilize variances (Draper and Smith, 1991). Transformations are expressed in terms of the untransformed variate *Y*. All analyses were undertaken using the SAS/STAT software package (version 6.12, SAS Institute Inc., Cary, NC).

Results

Settlement and metamorphosis of Herdmania curvata *are inhibited by non-geniculate coralline algae*

Responses of *Herdmania curvata* larvae to NCA were similar in experiments in which larvae had access to all three NCA species simultaneously (choice experiment; Fig. 1A), and those in which larvae were presented with only a single species (no choice experiment; Fig. 1B). In control cultures containing seawater only, the majority of larvae (60.8% ±SE = 4.4% and 51.1% ±SE = 4.6% in the choice and no choice trials respectively) had settled and metamorphosed on the bottom of the plastic chamber 11–13 h after attaining competence (~14–16 h after hatching; Figs. 1A, 1B). These larvae were metamorphosing normally; *i.e.*, tail muscle cells were in the process of being degraded, ampullae and the postlarval tunic were forming, and the endodermal primordia had turned and commenced morphogenesis. Larvae that had not settled or metamorphosed were also normal and possessed extended sensory papillae.

In contrast, in treatments containing NCA the majority of larvae (*ca.* 80%) neither settled nor metamorphosed (Fig. 1), no postlarvae settled on the epithallial surface of any of the NCA, and the low levels of settlement (*ca.* 20%) that did occur were confined wholly to the periphery of the plastic chambers. Although we did not quantify larval behavior during the experiment, we did observe some larvae directly contacting the NCA, and we noted that larvae exhibited their usual weak and irregular swimming behavior. Although early metamorphosis appeared normal for *H. curvata* settled on the plastic in the presence of NCA (*i.e.*, larval tail resorption, papillae retraction, and initiation of the programmed degradation of muscle myofibrils appeared normal), metamorphosis was abnormal in that postlarvae did not develop ampullae or undergo the rotation of the endodermal primordium (see Degnan *et al.*, 1996, 1997). Most of the unsettled *H. curvata* larvae appeared abnormal (see below).

By exposing larvae to different quantities of NCA, significant differences were observed in larval responses to the different coralline species, and to different quantities of any given species (Fig. 2). For a given amount of NCA, larval settlement was consistently (and often significantly) greater in the presence of *Lithothamnium prolifer* than either of the other species. Larvae showed greatest sensitivity to *Neogoniolithon brassica-florida*, and the inhibitory effect increased dramatically when the percentage of the base of the settlement chamber that was covered with shards increased from small (5%–15% coverage) to medium (25%–35% coverage). Of these treatments, normal metamorphosis occurred only in *H. curvata* cultured in the presence of small and medium shards of *L. prolifer*. Overall, the inhibitory effect on larval settlement was ranked *N. brassica-florida* > *Hydrolithon onkodes* > *L. prolifer*.

Non-geniculate coralline algae cause sloughing of larval sensory papillae and necrotic cell death

Unlike normal 14–16 h posthatch larvae, which have extended papillae, larvae cultured in the presence of NCA had rounded trunks and lacked papillae (Fig. 3). Normal larvae were evident only in the treatment containing small and medium shards of *L. prolifer* (Fig. 2A). Microscopic inspection of larvae revealed that all three NCA species appeared to induce this same effect on larval morphology, although the amount of NCA required to affect larval morphology differed (Fig. 2A). For these reasons, only the effects of *L. prolifer*, the least potent of the NCA, on *H. curvata* larvae were documented further.

Large shards of *L. prolifer* (Fig. 2A) induced a range of changes in *H. curvata* larval morphology that appeared to be related but differed in severity. Slightly abnormal larvae had normal axial structures (notochord, neural tube, and muscle) and trunk structures (sensory vesicle containing otolith and ocellus, and endoderm rudiment) but lacked projecting papillae and had a slightly rounded trunk (Fig. 3C, D). Some of these larvae still had papillae associated with them; however, the papillae were no longer attached to the anterior trunk and appeared to be in the process of being sloughed off (Fig. 3E). In the most severely altered larvae, the trunk was small and rounded, the tail was kinked, and the muscle cells had lost integrity and their usual columnar shape (Fig. 3F). Between these extremes of NCA-induced abnormalities was a continuum of morphological defects.

To determine whether the inhibitory factor or factors associated with *L. prolifer* or the other NCA were inducing general cell death, we investigated the structure of the nuclei of larvae that exhibited an intermediate abnormal morphology (*i.e.*, rounded trunk, straight tail with slightly rounded muscle cells). Nuclei were stained with propidium iodide and analyzed by laser scanning confocal microscopy. Optical sections were taken through the trunk epidermis and mesenchyme of normal and abnormal larvae, and compared. In both tissues, the nuclei of larvae exposed to *L. prolifer* were larger than those of normal larvae (Fig. 4). Mesenchymal nuclei of normal larvae were circular (diameters between 3.5 and 4.3 μm) and appeared granular when stained

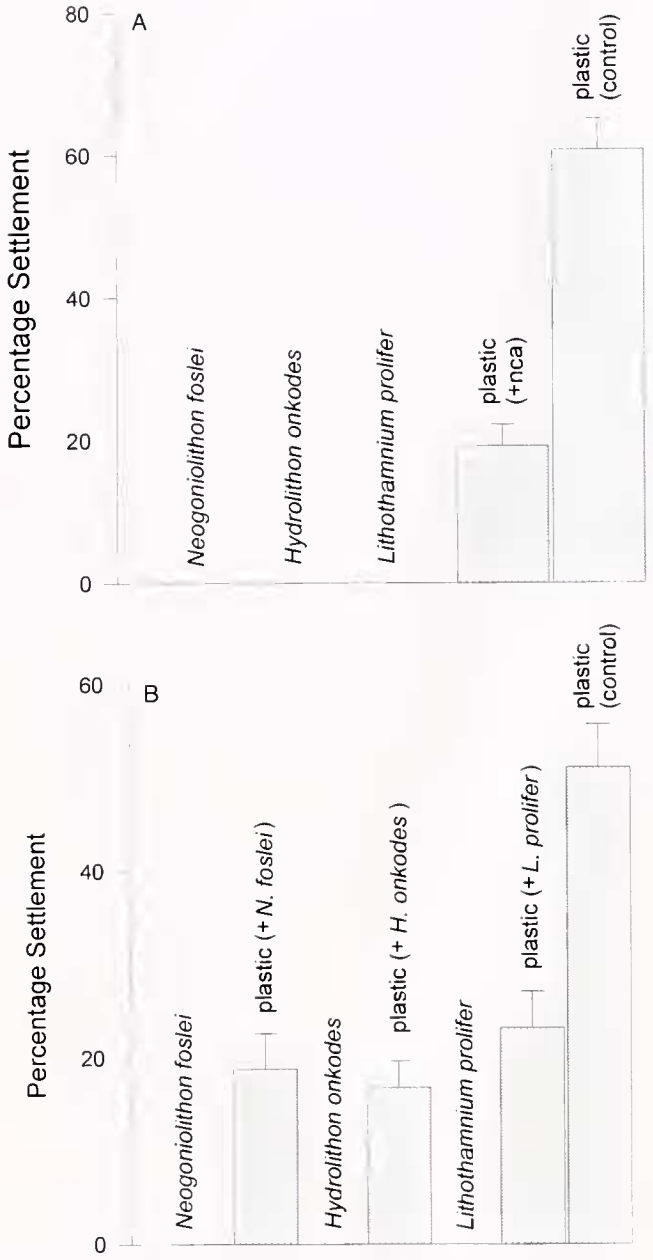

Figure 1. Inhibition of *Herdmania curvata* settlement by non-geniculate coralline algae (NCA). Results are mean percentage settlement (+SE) of *H. curvata* larvae on three species of NCA (*Neogoniolithon brassica-florida*, *Hydrolithon onkodes*, *Lithothamnium prolifer*), on the sides of settlement chambers containing NCA, and on the sides of chambers in the control treatment (seawater only). (A) "Choice" experiment in which larvae were presented with all species of NCA simultaneously. No larvae settled on any NCA, and settlement rates on the sides of settlement chambers were significantly lower in chambers containing NCA ($n = 15$) than in control chambers ($n = 15$) containing seawater only (one-way ANOVA, $F(1, 28) = 61.7$, $P < 0.0001$, no transformation required). (B) "No choice" experiment in which larvae were exposed to only a single species of NCA. No larvae settled on any NCA, and settlement rates on the sides of settlement chambers were significantly lower in chambers containing NCA ($n = 10$) than in control chambers ($n = 9$) containing seawater only (one-way ANOVA, $F(3, 35) = 16.9$, $P < 0.0001$, no transformation required). Settlement rates on the sides of chambers did not differ among NCA treatments [Tukey's groupings at $P = 0.05$; control > (*N. brassica-florida* = *H. onkodes* = *L. prolifer*)].

with propidium iodide (Fig. 4A). The mesenchymal nuclei of larvae cultured with *L. prolifer* were oval, larger (4.3–7.1 μm), and more diffusely stained with propidium iodide (Fig. 4B). Normal epidermal nuclei were similar in appearance to normal mesenchymal nuclei (Fig. 4C). Larvae exposed to *L. prolifer* had irregularly shaped epidermal nuclei that stained intensely with propidium iodide and were about the same size as normal nuclei (Fig. 4D). There was additional, non-nuclear staining of these epidermal cells.

Metamorphosis cannot occur in larvae previously exposed to non-geniculate coralline algae

To determine whether the effect of the NCA on *H. curvata* larvae was transient, we transferred larvae that were cultured with either *H. onkodes*, *N. brassica-florida* or *L. prolifer* into culture chambers containing 40 m*M* KCl-elevated FSW. Because the larvae exhibited a range of abnormalities, we transferred only those showing slight abnormalities (*i.e.*, lost papillae and rounded trunk; *e.g.*, Fig. 3C, D). Larvae from the same fertilization batch that were not exposed to NCA and had not metamorphosed in the plastic chambers acted as controls and were transferred to the KCl-elevated FSW or FSW. We monitored and scored the number of larvae that had initiated metamorphosis (*i.e.*, began tail resorption) every hour for 3 h. Although most of the untreated larvae in both FSW and KCl-elevated FSW began metamorphosing over this period, only 2 of a total of 180 larvae previously exposed to any one of the algae initiated metamorphosis (Fig. 5). Analysis of these cultures after 24 h revealed that control larvae were metamorphosing normally and that larvae previously cultured with NCA had not metamorphosed and died. The two post-larvae previously exposed as larvae to the NCA were also dead after 24 h.

Discussion

Most competent *Herdmania curvata* larvae will normally settle and metamorphose in seawater, FSW, and FSW with antibiotics within 24 h of hatching (Degnan *et al.*, 1997; unpub. data). The percentage of larvae that will spontaneously settle varies between cohorts, with those cultured in unfiltered seawater generally settling at a greater rate than those cultured in FSW. To assess the extent of any inhibitory effects of NCA on larval settlement and metamorphosis, we cultured the larvae and NCA in unfiltered seawater. The high percentage of larvae that settled in chambers containing seawater demonstrated that *H. curvata* will settle spontaneously under these culture conditions. The significant reduction in settlement rates of larvae cultured in the presence of the different NCA demonstrates that these algae are inhibiting settlement in this tropical ascidian.

H. curvata larvae respond differentially to a range of epifloral and faunal substrata associated with the cryptic

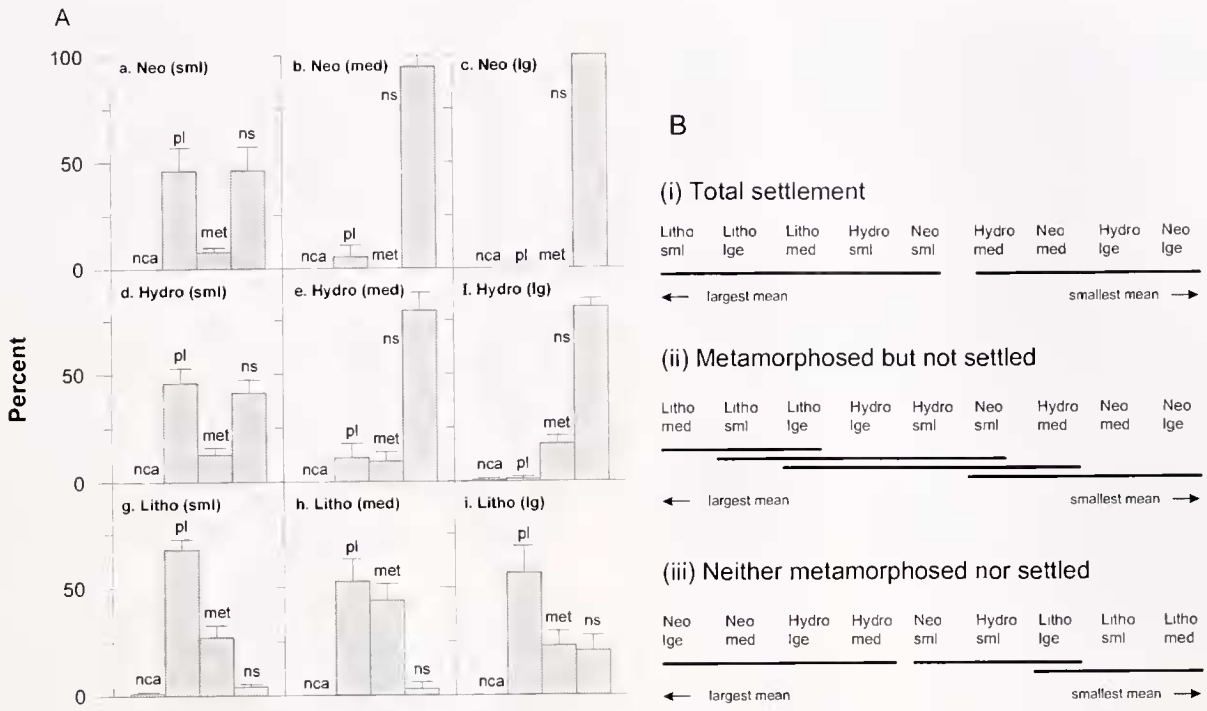

Figure 2. Dose-dependence of inhibitory effect of non-geniculate coralline algae on settlement and metamorphosis of *Herdmania curvata* in a "no-choice" experiment. (A) Mean response (+SE) of *H. curvata* larvae in the presence of small, medium, and large (= "sml," "med," "lge," respectively) shards of the NCA *Neogoniolithon brassica-florida* (= "Neo"), *Hydrolithon onkodes* (= "Hydro"), and *Lithothamnium prolifer* (= "Litho"). "nca" = settlement and metamorphosis on NCA shards; "pl" = settlement and metamorphosis on the sides of the plastic settlement chambers; "met" = larvae metamorphosed but not settled, and "ns" = larvae neither settled or metamorphosed; $n = 6$ for all treatments. Greatest total settlement occurred in controls (68.72% ± SE = 5.11; not shown on figure). Tukey groupings ($P = 0.05$) indicated settlement in controls was significantly greater than in treatments containing medium- and large-sized shards of *N. brassica-florida* and *H. onkodes* (one-way ANOVA, $F(9, 50) = 15.05$, $P < 0.0001$, transformation $\gamma^{0.553}$). The pattern of settlement in control chambers ($n = 6$) containing seawater only (not shown) was not significantly different from the treatment containing small shards of *L. prolifer* (panel g). (B) Tukey groupings following detection of significant interaction between NCA species and size of shard (= "dose") for total larvae settled, larvae metamorphosed but not settled, and larvae neither settled nor metamorphosed (two-way ANOVA, species × size interaction: total larvae settled and metamorphosed, $F(4, 45) = 3.31$, $P = 0.018$, transformation $\gamma^{0.553}$; larvae metamorphosed but not settled, $F(4, 45) = 5.02$, $P = 0.002$, transformation = arcsin $\sqrt{\gamma}$; larvae not settled or metamorphosed, $F(4, 45) = 4.96$, $P = 0.002$, no transformation required). These results show that the inhibitory effect of *N. brassica-florida* and *H. onkodes* on larval settlement and metamorphosis was greater than that of *L. prolifer*, and that the dose-response relationship was significantly steeper for *N. brassica-florida* and *H. onkodes* than for *L. prolifer*.

community of the reef crest and slope of the Great Barrier Reef: metamorphosis is induced by some substrata and not induced or inhibited by others in the laboratory. Manual removal of the larval trunk anterior of the otolith and ocellus prevents the posterior part of the larva from being induced to metamorphose with KCl-elevated FSW or natural inducers (Degnan *et al.*, 1997), suggesting that responsiveness to inductive substrata in *H. curvata* is mediated by the chemosensory papillae and an anterior signaling center. The *Hemps* gene, which encodes a protein with a putative secretion signal sequence and epidermal growth factor (EGF)-like repeats, is expressed in this region and has been shown to regulate the induction of metamorphosis (Arnold *et al.*, 1997; Eri *et al.*, 1999). The NCA investigated in this study (*Neogoniolithon brassica-florida, Hydrolithon onkodes*, and *Lithothamnium prolifer*) appear to be toxic to *H. curvata* larvae, inhibiting settlement on the surface of the algae, significantly lowering the level of spontaneous settlement, and preventing the future ability of larvae to respond to inductive cues. Trunk ectodermal and mesenchymal nuclei of larvae cultured in the presence of *L. prolifer* were bloated and irregular in shape respectively, both features of necrotic cell death (Kerr and Harmon, 1991). Induced morphogenetic changes during normal metamorphosis do not include this form of cell death (see Degnan *et al.*, 1996, 1997; Hinman and Degnan, 1998). *H. curvata* larvae that were

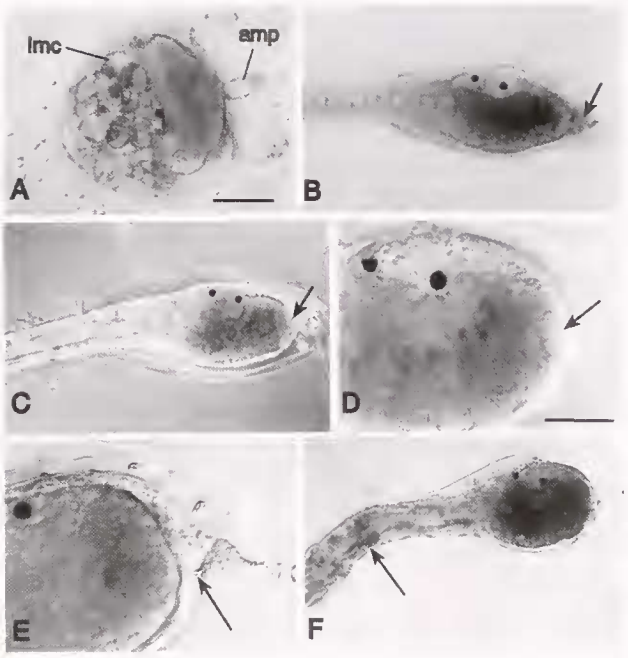

Figure 3. The effect of *Lithothamnium prolifer* on normal development and larval structures of *Herdmania curvata*. Larval anterior is to the right in all micrographs. (A) A normal postlarva approximately 12 h after initiating metamorphosis; degenerating larval muscle cells (lmc) and projecting ampullae (amp) are evident. (B) Normal tadpole larva with sensory papillae (arrow). (C–F) Larvae cultured with *L. prolifer*. (C, D) Larva with a rounded trunk and no papillae; arrows point to region where papillae are normally located. (E) Larva in the process of shedding a papilla (arrow). (F) Larva with rounded trunk, no papillae, kinked tail, and necrosing muscle cells (arrow). Scale bars: A, B, C, F, 100 μm); D, E, 50 μm.

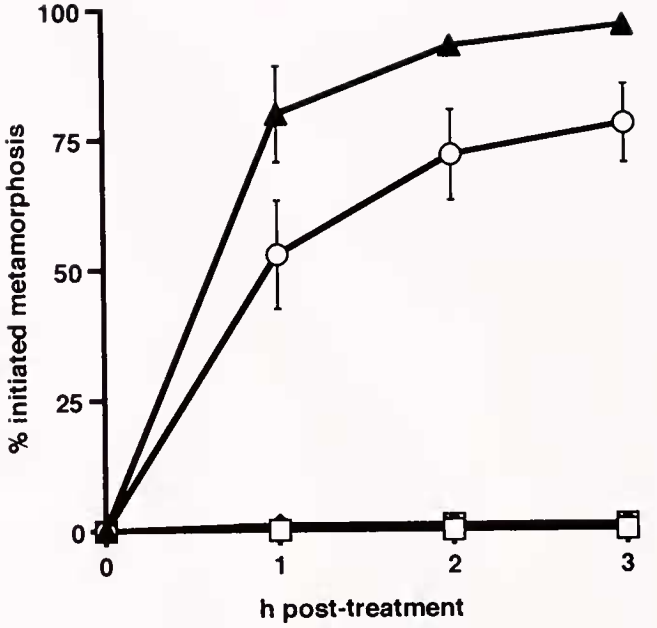

Figure 5. Percentage of *Herdmania curvata* larvae metamorphosing when treated with 40 m*M* KCl-elevated FSW. Prior to treatment with KCl-elevated FSW, larvae were cultured either in FSW, or in the presence of *Neogoniolithon brassica-florida*, *Hydrolithon onkodes*, or *Lithothamnium prolifer* for 12–14 h. Filled triangles, larvae cultured in seawater and then transferred to KCl-elevated FSW; open circles, larvae cultured in seawater and then transferred to FSW; open squares, larvae exposed to *Neogoniolithon brassica-florida* and then transferred to KCl-elevated FSW; diamonds (hidden behind open squares), larvae exposed to *Hydrolithon onkodes* and then transferred to KCl-elevated FSW; half-filled squares (hidden behind open squares), larvae exposed to *Lithothamnium prolifer* and then transferred to KCl-elevated FSW. Data are means (±SE).

cultured with NCA and had not settled lacked papillae and had rounded anterior trunks. In some of the least morphologically disturbed individuals, the palps were observed being sloughed from the trunk of the larva, suggesting that the toxic effect of the NCA upon the larva first disables the chemosensory and primary signaling system. *Hemps* is expressed in the papillae and the papillae-associated tissue (PAT) which is located in the anterior epidermis between the three papillae and consists of about 5 cells (Eri *et al.*, 1999). In the vicinity of the sloughed papillae are individual cells that may have been part of PAT (Fig. 3E). Secretion of the Hemps protein is essential for the induction and progression of metamorphosis (Eri *et al.*, 1999). It is possible that PAT cells are exuded precociously in larvae exposed to NCA, disrupting the Hemps signaling system. *H. curvata* larvae do not have the ability to regenerate these papillae nor regain the ability to respond to artificial (Fig. 5) or natural cues (data not shown). The global toxic effect of the NCA upon *H. curvata* larvae is demonstrated by the high percentage of death within 24 h of larvae being cultured with NCA. Experimental inhibition of papillae morphogenesis by ecotopic application of retinoic acid results in a very similar phenotype to larvae cultured with NCA; however, metamorphosis is not inhibited (Hinman and Degnan, 1998). Toxicity is not limited to the larvae—individuals that

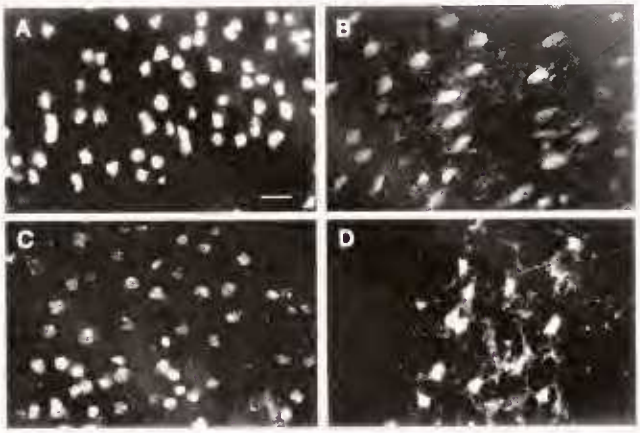

Figure 4. Degeneration of the nuclei of *Herdmania curvata* larvae cultured in the presence of *Lithothamnium prolifer*. Confocal micrographs of nuclei stained with propidium iodide. (A) Normal larval trunk mesenchyme (trunk ventral cells; Satoh, 1996). (B) Trunk mesenchyme of larva exposed to *L. prolifer*. (C) Normal larval trunk epidermis. (D) Trunk epidermis of larva exposed to *L. prolifer*. Scale bar, 10 μm.

did settle and initiate metamorphosis in these assays did not complete metamorphosis and died within 24 h (data not shown).

The inhibitory signals produced by these NCA appear to be taxa specific. Importantly, under similar laboratory conditions, we have induced normal settlement and metamorphosis of an asteroid (with *L. prolifer;* Johnson *et al.,* 1991; Johnson and Sutton, 1994) and observed that coral and mollusc larvae are not affected by these algae under identical assay conditions (unpub. data). Given that the effect of each of the NCA species on *H. curvata* larval morphology was very similar (*i.e.,* all induced rounded trunks and sometimes kinked tails and dissociated muscle cells), a similar inhibitory factor may be being produced by all three species of NCA. We did not determine whether the inhibitors were produced by the NCA or by surface-associated bacteria or microalgae.

Although it is well established that NCA provide morphogenic cues for larvae of a variety of marine invertebrates, but do not induce settlement or metamorphosis in others (see Introduction), this report demonstrates toxic inhibition of settlement and metamorphosis of an invertebrate by NCA. The distinction between the simple absence of a morphogenic cue and inhibition is important. Although larvae will not settle and metamorphose in either case, if the initial contact is with a substratum that simply lacks an inductive cue, the larva can continue to search and may subsequently receive the appropriate stimulus to settle and metamorphose. These NCA are apparently not toxic to other larvae, since some coral and mollusc larvae swim in the assay chambers and behave normally for several days without settling (unpub. data). Importantly, the ability of *H. curvata* larvae to undergo high rates of "spontaneous" settlement and metamorphosis in seawater (Degnan *et al.,* 1997) or when exposed to a potent inducer (KCl) allows us to determine whether a substratum is actually inhibiting settlement and metamorphosis or is merely not providing a morphogenic cue. If the ascidian larva contacts an inhibitory substratum—in this case three species of NCA—then lower (or zero) rates of settlement or metamorphosis will be detected, even when the larvae are subsequently exposed to a potent inducer (KCl). In contrast, because many larvae do not readily undergo spontaneous metamorphosis (*e.g.,* the mollusc and coral used in the unpublished assays), it is not possible to determine whether the NCA are inhibiting settlement by means of a nontoxic pathway or are simply having no effect on the larvae.

If the inhibitory effect reported here occurs in nature as it does in the laboratory, then given extensive cover of NCA in the preferred habitat of *H. curvata* (shallow water on the reef crest), this interaction is likely to have a large effect in determining the small-scale distribution of *H. curvata.* Although the inhibitory effect is evident when larvae are prevented from contacting the surface of the NCA (unpub. data), indicating that the inhibitors can be released from the surface, it is likely that the inhibitors would operate on a microscopic scale because of dilution to noneffective concentrations a short distance from the NCA. The inability of NCA to induce settlement and metamorphosis in *H. curvata* is consistent with a general pattern that NCA induce settlement and metamorphosis in motile species whose feeding activities reduce fouling of the plant surface, and in sessile species that provide preferred habitat for NCA, but not in sessile species that potentially grow to smother and kill NCA (C. R. Johnson, unpub. manuscript).

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Literature Cited

Arnold, J. M., R. Eri, B. M. Degnan, and M. F. Lavin. 1997. A novel gene containing multiple EGF-like motifs transiently expressed in the papillae of the ascidian tadpole larva. *Dev. Dyn.* **210:** 264–273.

Barnes, J. R., and J. J. Gonor. 1973. The larval settling response of the lined chiton *Tonicella lineata. Mar. Biol.* **20:** 259–264.

Cloney, R. A. 1982. Ascidian larvae and events of metamorphosis. *Am. Zool.* **22:** 817–826.

Coon, S. L., W. K. Fitt, and D. B. Bonar. 1990. Competence and delay of metamorphosis in the Pacific oyster *Crassostrea gigas. Mar. Biol.* **106:** 379–387.

Davis, A. R., N. M. Targett, O. J. McConnell, and C. M. Young. 1989. Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth. *Bioorg. Mar. Chem.* **3:** 85–114.

Degnan, B. M., and M. F. Lavin. 1995. Highly repetitive DNA sequences provide evidence for a lack of gene flow between morphological forms of *Herdmania momus. Mar. Biol.* **124:** 293–299.

Degnan, B. M., and D. E. Morse. 1995. Developmental and morphogenetic gene regulation in *Haliotis rufescens* larvae at metamorphosis. *Am. Zool.* **35:** 391–398.

Degnan, B. M., J. C. Groppe, and D. E. Morse. 1995. Chymotrypsin mRNA expression in digestive gland amoebocytes: cell specification occurs prior to metamorphosis and gut morphogenesis in the gastropod *Haliotis rufescens. Roux's Arch. Dev. Biol.* **205:** 97–101.

Degnan, B. M., P. Rohde, and M. F. Lavin. 1996. Normal development and embryonic gene activity of the ascidian *Herdmania momus. Mar. Freshw. Res.* **47:** 543–551.

Degnan, B. M., D. Souter, S. M. Degnan, and S. C. Long. 1997. Induction of metamorphosis with potassium ions requires development of competence and an anterior signalling center in the ascidian *Herdmania momus. Dev. Genes Evol.* **206:** 370–376.

De Silva, P. H. D. H. 1962. Experiments on choice of substrate by *Spirorbis* larvae (Serpulidae). *J. Exp. Biol.* **39:** 483–490.

Draper, N. R., and H. Smith. 1991. *Applied Regression Analysis.* Wiley, New York. 709 pp.

Eri, R., J. M. Arnold, V. F. Hinman, K. M. Green, M. K. Jones, B. M. Degnan, and M. F. Lavin. 1999. Hemps, a novel EGF-like protein, plays a central role in ascidian metamorphosis. *Development* **126** (in press).

Fitt, W. K., M. P. Labare, W. C. Fuqua, M. Walch, S. L. Coon, D. B. Bonar, R. R. Colwell, and R. M. Weiner. 1989. Factors influencing bacterial production of inducers of settlement behaviour of larvae of the oyster *Crassostrea gigas*. *Microb. Ecol.* **17:** 287–298.

Gee, J. M. 1965. Chemical stimulation of settlement in larvae of *Spirorbis rupestris* (Serpulidae). *Anim. Behav.* **13:** 181–186.

Gee, J. M., and E. W. Knight-Jones. 1962. The morphology and larval behaviour of a new species of *Spirorbis* (Serpulidae). *J. Mar. Biol. Assoc. UK* **42:** 641–654.

Hadfield, M. G. 1986. Settlement and recruitment of marine invertebrates: a perspective and some proposals. *Bull. Mar. Sci.* **39:** 418–425.

Hadfield, M. G., and J. T. Pennington. 1990. Nature of metamorphic signal and its internal transduction in larvae of the nudibranch *Phestilla sibogae*. *Bull. Mar. Sci.* **46:** 455–464.

Hahn, K. O. 1989. Induction of settlement in competent abalone larvae. Pp. 101–112 in *Handbook of Culture of Abalone and Other Marine Gastropods*, K. O. Hahn, ed. CRC Press, Florida.

Harrigan, J. F. 1972. Behavior of the planula larva of the scleractinian coral *Pocillopora damicornis* (L.). *Am. Zool.* **12:** 723.

Heslinga, G. A. 1981. Larval development, settlement and metamorphosis of the tropical gastropod *Trochus niloticus*. *Malacologia* **20:** 349–357.

Hinman, V. F., and B. M. Degnan. 1998. Retinoic acid disrupts ectodermal and endodermal development in ascidian larvae and postlarvae. *Dev. Genes Evol.* **208:** 336–345.

Jensen, R. A., and D. E. Morse. 1984. Intraspecific facilitation of larval recruitment: gregarious settlement of the polychaete *Phragmatopoma californica* (Fewkes). *J. Exp. Mar. Biol. Ecol.* **83:** 107–126.

Johnson, C. R., and D. C. Sutton. 1994. Bacteria on the surface of crustose coralline algae induce metamorphosis of crown-of-thorns starfish. *Mar. Biol.* **120:** 305–310.

Johnson, C. R., D. C. Sutton, R. R. Olson, and R. Giddins. 1991. Settlement of crown-of-thorns starfish: role of bacteria on surfaces of crustose coralline algae and a hypothesis for deepwater recruitment. *Mar. Ecol. Prog. Ser.* **71:** 143–162.

Johnson, C. R., T. E. Lewis, D. S. Nichols, and B. M. Degnan. 1997. Bacterial induction of settlement and metamorphosis in marine invertebrates. *Proc. 8th Int. Coral Reef Symp.* **2:** 1219–1224.

Keough, M. J., and P. T. Raimondi. 1995. Responses of settling invertebrate larvae to bioorganic films: effects of different types of films. *J. Exp. Mar. Biol. Ecol.* **185:** 235–253.

Kerr, J. F. R., and B. V. Harmon. 1991. Definition and incidence of apoptosis: an historical perspective. Pp. 5–30 in *Apoptosis. The Molecular Basis of Cell Death*, L. D. Tomei and F. O. Cope, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Kirchman, D., S. Graham, D. Reish, and R. Mitchell. 1982. Bacteria induce settlement and metamorphosis of *Janua* (Dexiospira) *brasiliensis* Grube (Polychaeta: Spiorbidae). *J. Exp. Mar. Biol. Ecol.* **56:** 153–163.

Leitz, T. 1997. Induction of settlement and metamorphosis of cnidarian larvae: signals and signal transduction. *Invertebr. Reprod. Dev.* **31:** 109–122.

Maki, J. S., D. Rittschof, J. D. Costlow, and R. Mitchell. 1988. Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial surface films. *Mar. Biol.* **97:** 199–206.

Maki, J. S., D. Rittschof, M. O. Samuelsson, U. Szewzyk, A. B. Yule, S. Kjelleberg, J. D. Costlow, and R. Mitchell. 1990. Effect of marine bacteria and their exopolymers on the attachment of barnacle cypris larvae. *Bull. Mar. Sci.* **46:** 499–511.

Maki, J. S., D. Rittschof, and R. Mitchell. 1992. Inhibition of larval barnacle attachment to bacterial films: an investigation of physical properties. *Microb. Ecol.* **23:** 97–106.

Morse, A. N. C., and D. E. Morse. 1984. Recruitment and metamorphosis of *Haliotis* larvae induced by molecules uniquely available at the surfaces of the crustose red algae. *J. Exp. Mar. Biol. Ecol.* **75:** 191–215.

Morse, D. E. 1990. Recent progress in larval settlement and metamorphosis: closing the gap between molecular biology and ecology. *Bull. Mar. Sci.* **46:** 465–483.

Morse, D. E. 1993. Signalling in planktonic larvae. *Nature* **363:** 406.

Morse, D. E., N. Hooker, A. N. C. Morse, and R. A. Jensen. 1988. Control of larval metamorphosis and recruitment in sympatric agariciid corals. *J. Exp. Mar. Biol. Ecol.* **116:** 193–217.

Moss, G. A., and L. J. Tong. 1992. Effect of stage of larval development on the settlement of the abalone, *Haliotis iris*. *NZ J. Mar. Freshwater Res.* **26:** 69–73.

Pawlik, J. R. 1992. Chemical ecology of the settlement of benthic marine invertebrates. *Oceanogr. Mar. Biol. Annu. Rev.* **30:** 273–335.

Rowley, R. J. 1989. Settlement and recruitment of sea urchins (*Strongylocentrotus* spp.) in a sea-urchin barren ground and a kelp bed: are populations regulated by settlement or post-settlement processes? *Mar. Biol.* **100:** 485–494.

Rumrill, S. S., and R. A. Cameron. 1983. Effects of gamma-aminobutyric acid on the settlement of larvae of the black chiton *Katharina tunicata*. *Mar. Biol.* **72:** 243–247.

Satoh, N. 1994. *Developmental Biology of Ascidians.* Cambridge University Press, Cambridge. 234 pp.

Sebens, K. P. 1983a. Settlement and metamorphosis of a temperate soft-coral larva (*Alcyonium siderium* Verrill): induction by crustose algae. *Biol. Bull.* **165:** 286–304.

Sebens, K. P. 1983b. The larval and juvenile ecology of the temperate octocoral *Alcyonium siderium* Verrill. I. Substratum selection by benthic larvae. *J. Exp. Mar. Biol. Ecol.* **71:** 73–89.

Torrence, S. A., and R. A. Cloney. 1983. Ascidian larval nervous system: primary sensory neurons in adhesive papillae. *Zoomorphology* **102:** 111–123.

Trapido-Rosenthal, H. G., and D. E. Morse. 1986. Availability of chemosensory receptors is down regulated by habituation of larvae to a morphometric signal. *Proc. Natl. Acad. Sci. USA* **83:** 7658–7662.

Tritar, S., D. Prieur, and R. Weiner. 1992. Effects of bacterial films on the settlement of the oysters, *Crassostrea gigas* (Thunberg, 1793) and *Ostrea edulis*, Linnaeus, 1750 and the scallop *Pecten maximus* (Linnaeus, 1758). *J. Shellfish Res.* **11:** 325–330.

Wilson, D. P. 1955. The role of micro-organisms in the settlement of *Ophelia bicornis* Savigny. *J. Mar. Biol. Assoc. UK* **34:** 531–543.